

INGAP PROTEIN INVOLVED IN PANCREATIC ISLET NEOGENESIS

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BACKGROUND OF THE INVENTION

Pancreatic islets of Langerhans are the only organ of insulin production in the body. However, they have a limited capacity for regeneration. This limited regeneration capacity predisposes mammals to develop diabetes mellitus. Thus there is a need in the art of endocrinology for products which can stimulate the regeneration of islets of Langerhans to prevent or ameliorate the symptoms of diabetes mellitus.

One model of pancreatic islet cell regeneration involves cellophane-wrapping of the pancreas in the Syrian golden hamster (1). Wrapping of the pancreas induces the formation of new endocrine cells which appear to arise from duct epithelium (2-4). There is a need in the art to identify and isolate the factor(s) which is responsible for islet cell regeneration.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a preparation of a mammalian protein or polypeptide portions thereof involved in islet cell neogenesis.

It is another object of the invention to provide a DNA molecule encoding a mammalian protein involved in islet cell neogenesis.

It is yet another object of the invention to provide a preparation of a mammalian INGAP (islet neogenesis associated protein) protein.

It is still another object of the invention to provide nucleotide probes for detecting mammalian genes involved in islet cell neogenesis.

It is an object of the invention to provide a method for isolation of INGAP genes from a mammal.

It is another object of the invention to provide an antibody preparation which is specifically immunoreactive with an INGAP protein.

It is yet another object of the invention to provide methods of producing INGAP proteins.

It is an object of the invention to provide methods for treating diabetic mammals.

It is another object of the invention to provide methods for growing pancreatic islet cells in culture.

It is still another object of the invention to provide methods of enhancing the life span of pancreatic islet cells encapsulated in polycarbon shells.

It is an object of the invention to provide methods of enhancing the number of pancreatic islet cells in a mammal.

It is an object of the invention to provide transgenic mammals.

It is another object of the invention to provide genetically engineered mammals.

It is yet another object of the invention to provide methods of identifying individual mammals at risk for diabetes.

It is an object of the invention to provide methods of detecting INGAP protein in a sample from a mammal.

It is still another object of the invention to provide a method of treating isolated islet cells to avoid apoptosis.

It is another object of the invention to provide methods of treating mammals receiving islet cell transplants.

It is an object of the invention to provide a method of inducing differentiation of β cell progenitors.

It is an object of the invention to provide a method of identifying β cell progenitors.

It is another object of the invention to provide a method of treating a mammal with pancreatic endocrine failure.

It is an object of the invention to provide antisense constructs for regulating the expression of INGAP.

It is yet another object of the invention to provide a method for treating nesidioblastosis.

It is still another object of the invention to provide kits for detecting mammalian INGAP proteins.

It is an object of the invention to provide pharmaceutical compositions for treatment of pancreatic insufficiency.

These and other objects of the invention are provided by one or more of the embodiments described below.

In one embodiment a preparation of a mammalian INGAP protein is provided. The preparation is substantially free of other mammalian proteins.

In another embodiment an isolated cDNA molecule is provided. The cDNA molecule encodes a mammalian INGAP protein.

In still another embodiment of the invention a preparation of a mammalian INGAP protein is provided. The preparation is made by the process of:

inducing mammalian pancreatic cells to express INGAP protein by cellophane-wrapping; and

purifying said INGAP protein from said induced mammalian pancreatic cells.

In yet another embodiment of the invention a nucleotide probe is provided. The probe comprises at least 20 contiguous nucleotides of the sequence shown in SEQ ID NO: 1.

In another embodiment of the invention a preparation of INGAP protein of a mammal is provided. The preparation is substantially purified from other proteins of the mammal. The INGAP protein is inducible upon cellophane-wrapping of pancreas of the mammal.

In yet another embodiment of the invention a method of isolating an INGAP gene from a mammal is provided. The method comprises:

hybridizing one or more oligonucleotides comprising at least 10 contiguous nucleotides of the sequence shown in SEQ ID NO: 1 to genomic DNA or cDNA of said mammal;

identifying DNA molecules from said genomic DNA or cDNA which hybridize to said one or more oligonucleotides.

In still another embodiment of the invention an isolated cDNA molecule is provided. The cDNA molecule is obtained by the process of:

hybridizing one or more oligonucleotides comprising at least 10 contiguous nucleotides of the sequence shown in SEQ ID NO: 1 to genomic DNA or cDNA of said mammal;

identifying DNA molecules from said genomic DNA or cDNA which hybridize to said one or more oligonucleotides.

In another embodiment of the invention an antibody is provided. The antibody is specifically immunoreactive with a mammalian INGAP protein.

According to still another embodiment of the invention a method of producing a mammalian INGAP protein is provided. The method comprises the steps of:

providing a host cell transformed with a cDNA encoding a mammalian INGAP protein;

identifying a mutation in an INGAP gene of a sample of an individual mammal, said mutation causing a structural abnormality in an INGAP protein encoded by said gene or causing a regulatory defect leading to diminished or obliterated expression of said INGAP gene.

According to another embodiment of the invention a kit for detecting a mammalian INGAP protein in a sample from a mammal is provided. The kit comprises:

an antibody preparation which is specifically immunoreactive with a mammalian INGAP protein; and

a polypeptide which comprises a sequence of at least 15 consecutive amino acids of a mammalian INGAP protein.

According to another embodiment of the invention a pharmaceutical composition for treatment of pancreatic insufficiency is provided. The composition comprises:

a mammalian INGAP protein in a pharmaceutically acceptable diluent or carrier.

According to another embodiment of the invention a pharmaceutical composition is provided. The composition comprises:

a preparation of a polypeptide which comprises a sequence of at least 15 consecutive amino acids of a mammalian INGAP protein and a pharmaceutically acceptable diluent or carrier.

These and other embodiments of the invention provide the art with means of stimulating and inhibiting islet cell neogenesis. Means of diagnosis of subsets of diabetes mellitus are also provided by this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B. Nucleotide sequence of hamster INGAP and deduced sequence of encoded immature protein (SEQ ID NOS: 1 and 2). The non-coding sequences are in lower case letters, and the polyadenylation signal is underlined.

FIG. 2. Comparison of amino acid sequences of INGAP (SEQ ID NO: 2), rat PAP-I (PAP-I)(18)(SEQ ID NO: 3), Human PAP/HIP (PAP-H/HIP)(10,11)(SEQ ID NO: 4), rat PAP-III (PAP-III)(9)(SEQ ID NO: 5), rat PAP-II (PAP-II)(8)(SEQ ID NO: 6), Rat Reg/PSP/Lithostatine (REG/LITH)(13,15)(SEQ ID NO: 7) and the invariable motif found by Drickamer in all members of C-type lectins (Drickamer)(12). Six conserved cysteines are marked by asterisks and the 2 putative N-glycosylation sites of INGAP are underlined and in bold letters.

FIGS. 3A, 3B and 3C. Northern blot analysis of INGAP and amylase gene expression in pancreatic tissue from control and wrapped hamster pancreas. 30 g of heat denatured total RNA was separated by electrophoresis on a 1.2% agarose, 0.6% formaldehyde/MOPS denaturing gel, and transferred to nylon membrane. Membranes were hybridized with a 747 bp hamster INGAP cDNA probe (cloned in our lab) (A), a 1000 bp rat amylase cDNA probe (generously given by Chris Newgard Dallas, Tex.) (B) and with an 18S ribosomal 24mer synthetic oligonucleotide probe to control for RNA integrity and loading (C).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We now report the identification of a gene, INGAP, that shows striking homology to the pancreatitis associated protein (PAP) family of genes (7-11). The predicted protein shares the carbohydrate recognition domain (CRD) of the calcium dependent C-type lectins as defined by Drickamer (12). INGAP protein plays a role in stimulation of islet neogenesis, in particular, in beta cell regeneration from ductal cells.

The cDNA sequence of a mammalian INGAP is provided in SEQ ID NO: 1. The predicted amino acid sequence is shown in SEQ ID NO:2. These sequences were determined from nucleic acids isolated from hamster, but it is believed that other mammalian species will contain INGAP genes

which are quite similar. For example, human INGAP cDNA shares the entire sequence in SEQ ID NO:1 with the hamster. The predicted amino acid sequence of human INGAP protein is from 1 to 174 in SEQ ID NO:2. One would expect homologous genes to contain at least about 70% identity. Closer species would be expected to have at least about 75%, 80%, 85%, 90%, 95%, or even 99% identity. In contrast, other family members of the calcium dependent C-type lectins contain at most 60% identity with INGAP.

The DNA sequence provided herein can be used to form vectors which will replicate the gene in a host cell, and may also express INGAP protein. DNA sequences which encode the same amino acid sequence as shown in SEQ ID NO:2 can also be used, without departing from the contemplation of the invention. DNA sequences coding for other mammalian INGAPs are also within the contemplation of the invention. Suitable vectors, for both prokaryotic and eukaryotic cells, are known in the art. Some vectors are specifically designed to effect expression of inserted DNA segments downstream from a transcriptional and translational control site. One such vector for expression in eukaryotic cells employs EBNA His, a plasmid which is available commercially from InVitrogen Corp. The loaded vector produces a fusion protein comprising a portion of a histidine biosynthetic enzyme and INGAP. Another vector, which is suitable for use in prokaryotic cells, is pcDNA3. Selection of a vector for a particular purpose may be made using knowledge of the properties and features of the vectors, such as useful expression control sequences. Vectors may be used to transform or transfect host cells, either stably or transiently. Methods of transformation and transfection are known in the art, and may be used according to suitability for a particular host cell. Host cells may be selected according to the purpose of the transfection. A suitable prokaryotic host is *E. coli* DH5 α . A suitable eukaryotic host is cos7, an African Green Monkey kidney cell line. For some purposes, proper glycosylation of INGAP may be desired, in which case a suitable host cell should be used which recognizes the glycosylation signal of INGAP.

Probes comprising at least 10, 15, 20, or 30 nucleotides of contiguous sequence according to SEQ ID NO:1 can be used for identifying INGAP genes in particular individuals or in members of other species. Appropriate conditions for hybridizations to same or different species' DNA are known in the art as high stringency and low stringency, respectively. These can be used in a variety of formats according to the desired use. For example, Southern blots, Northern blots, and in situ colony hybridization, can be used as these are known in the art. Probes typically are DNA or RNA oligomers of at least 10, 15, 20, or 30 nucleotides. The probe may be labeled with any detectable moiety known in the art, including radiolabels, fluorescent labels, enzymes, etc. Probes may also be derived from other mammalian INGAP gene sequences.

INGAP genes can be isolated from other mammals by utilizing the nucleotide sequence information provided herein. (More laboriously, they can be isolated using the same method described in detail below for isolation of the hamster INGAP gene.) Oligonucleotides comprising at least 10 contiguous nucleotides of the disclosed nucleotide sequence of INGAP are hybridized to genomic DNA or cDNA of the mammal. The DNA may conveniently be in the form of a library of clones. The oligonucleotides may be labelled with any convenient label, such as a radiolabel or an enzymatic or fluorescence label. DNA molecules which hybridize to the probe are isolated. Complete genes can be constructed by isolating overlapping DNA segments, for

example using the first isolated DNA as a probe to contiguous DNA in the library or preparation of the mammal's DNA. Confirmation of the identity of the isolated DNA can be made by observation of the pattern of expression of the gene in the pancreas when subjected to cellophane wrapping, for example. Similarly, the biological effect of the encoded product upon pancreatic ductal cells will also serve to identify the gene as an INGAP gene.

If two oligonucleotides are hybridized to the genomic DNA or cDNA of the mammal then they can be used as primers for DNA synthesis, for example using the polymerase chain reaction or the ligase chain reaction. Construction of a full-length gene and confirmation of the identity of the isolated gene can be performed as described above.

INGAP protein may be isolated according to the invention by inducing mammalian pancreatic cells to express INGAP protein by means of cellophane-wrapping. This technique is described in detail in reference no. 1 which is expressly incorporated herein. INGAP protein so produced may be purified from other mammalian proteins by means of immunoaffinity techniques, for example, or other techniques known in the art of protein purification. An antibody specific for a mammalian INGAP is produced using all, or fragments of, the amino acid sequence of an INGAP protein, such as shown in SEQ ID NO: 2, as immunogens. The immunogens can be used to identify and purify immunoreactive antibodies. Monoclonal or polyclonal antibodies can be made as is well known in the art. The antibodies can be conjugated to other moieties, such as detectable labels or solid support materials. Such antibodies can be used to purify proteins isolated from mammalian pancreatic cells or from recombinant cells. Hybridomas which secrete specific antibodies for an INGAP protein are also within the contemplation of the invention.

Host cells as described above can be used to produce a mammalian INGAP protein. The host cells comprise a DNA molecule encoding a mammalian INGAP protein. The DNA can be according to SEQ ID NO:1, or isolated from other mammals according to methods described above. Host cells can be cultured in a nutrient medium under conditions where INGAP protein is expressed. INGAP protein can be isolated from the host cells or the nutrient medium, if the INGAP protein is secreted from the host cells.

It has now been found that INGAP and fragments thereof are capable of inducing and stimulating islet cells to grow. Moreover, they are capable of inducing differentiation of pancreatic duct cells, and of allowing such cells to avoid the apoptotic pathway. Thus many therapeutic modalities are now possible using INGAP, fragments thereof, and nucleotide sequences encoding INGAP. Therapeutically effective amounts of INGAP are supplied to patient pancreata, to isolated islet cells, and to encapsulated pancreatic islet cells, such as in a polycarbon shell. Suitable amounts of INGAP for therapeutic purposes range from 1-150 $\mu\text{g/kg}$ of body weight or in vitro from 1-10,000 $\mu\text{g/ml}$. Optimization of such dosages can be ascertained by routine testing. Methods of administering INGAP to mammals can be any that are known in the art, including subcutaneous, via the portal vein, by local perfusion, etc.

Conditions which can be treated according to the invention by supplying INGAP include diabetes mellitus, both insulin dependent and non-insulin dependent, pancreatic insufficiency, pancreatic failure, etc. Inhibition of INGAP expression can be used to treat nesidioblastosis.

According to the present invention, it has now been found that a small portion of INGAP is sufficient to confer bio-

logical activity. A fragment of 20 amino acids of the sequence of SEQ ID NO: 2, from amino acid #103-#122 is sufficient to stimulate pancreatic ductal cells to grow and proliferate. The effect has been seen on a rat tumor duct cell line, a hamster duct cell line, a hamster insulinoma cell line, and a rat insulinoma cell line. The analogous portions of other mammalian INGAP proteins are quite likely to have the same activity. This portion of the protein is not similar to other members of the pancreatitis associated protein (PAP) family of proteins. It contains a glycosylation site and it is likely to be a primary antigenic site of the protein as well. This fragment has been used to immunize mice to generate monoclonal antibodies.

The physiological site of expression of INGAP has been determined. INGAP is expressed in acinar tissue, in the exocrine portion of the pancreas. It is not expressed in ductal or islet cells, i.e., the paracrine portion of the pancreas. Expression occurs within 24-48 hours of induction by means of cellophane wrapping.

Transgenic animals according to the present invention are mammals which carry an INGAP gene from a different mammal. The transgene can be expressed to a higher level than the endogenous INGAP genes by judicious choice of transcription regulatory regions. Methods for making transgenic animals are well known in the art, and any such method can be used. Animals which have been genetically engineered to carry insertions, deletions, or other mutations which alter the structure of the INGAP protein or regulation of expression of INGAP are also contemplated by this invention. The techniques for effecting these mutations are known in the art.

Diagnostic assays are also contemplated within the scope of the present invention. Mutations in INGAP can be ascertained in samples such as blood, amniotic fluid, chorionic villus, blastocyst, and pancreatic cells. Such mutations identify individuals who are at risk for diabetes. Mutations can be identified by comparing the nucleotide sequence to a wild-type sequence of an INGAP gene. This can be accomplished by any technique known in the art, including comparing restriction fragment length polymorphisms, comparing polymerase chain reaction products, nuclease protection assays, etc. Alternatively, altered proteins can be identified, e.g., immunologically or biologically.

The present invention also contemplates the use of INGAP antisense constructs for treating nesidioblastosis, a condition characterized by overgrowth of β cells. The antisense construct is administered to a mammal having nesidioblastosis, thereby inhibiting the overgrowth of β cells. An antisense construct typically comprises a promoter, a terminator, and a nucleotide sequence consisting of a mammalian INGAP gene. The INGAP sequence is between the promoter and the terminator and is inverted with respect to the promoter as it is expressed naturally. Upon expression from the promoter, an mRNA complementary to native mammalian INGAP is produced.

Immunological methods for assaying INGAP in a sample from a mammal are useful, for example, to monitor the therapeutic administration of INGAP. Typically an antibody specific for INGAP will be contacted with the sample and the binding between the antibody and any INGAP in the sample will be detected. This can be by means of a competitive binding assay, in which the incubation mixture is spiked with a known amount of a standard INGAP preparation, which may conveniently be detectably labeled. Alternatively, a polypeptide fragment of INGAP may be used as a competitor. In one particular assay format, the

antibodies are bound to a solid phase or support, such as a bead, polymer matrix, or a microtiter plate.

According to the present invention, pancreatic duct cells of a mammal with pancreatic endocrine failure can be removed from the body and treated *in vitro*. The duct cells typically comprise β cell progenitors. Thus treatment with a preparation of a mammalian INGAP protein will induce differentiation of the β cell progenitors. The duct cells are contacted with a preparation of a mammalian INGAP protein substantially free of other mammalian proteins. The treated cells can then be used as an autologous transplant into the mammal from whom they were derived. Such an autologous treatment minimizes adverse host versus graft reactions involved in transplants.

INGAP protein can also be used to identify those cells which bear receptors for INGAP. Such cells are likely to be the β cell progenitors, which are sensitive to the biological effects of INGAP. INGAP protein can be detectably labeled, such as with a radiolabel or a fluorescent label, and then contacted with a population of cells from the pancreatic duct. Cells which bind to the labeled protein will be identified as those which bear receptors for INGAP, and thus are β cell progenitors. Fragments of INGAP can also be used for this purpose, as can immobilized INGAP which can be used to separate cells from a mixed population of cells to a solid support. INGAP can be immobilized to solid phase or support by adsorption to a surface, by means of an antibody, or by conjugation. Any other means as is known in the art can also be used.

Kits are provided by the present invention for detecting a mammalian INGAP protein in a sample. This may be useful, *inter alia*, for monitoring metabolism of INGAP during therapy which involves administration of INGAP to a mammal. The kit will typically contain an antibody preparation which is specifically immunoreactive with a mammalian INGAP protein. The antibodies may be polyclonal or monoclonal. If polyclonal they may be affinity purified to render them monospecific. The kit will also typically contain a polypeptide which has at least 15 consecutive amino acids of a mammalian INGAP protein. The polypeptide is used to compete with the INGAP protein in a sample for binding to the antibody. Desirably the polypeptide will be detectably labeled. The polypeptide will contain the portion of INGAP to which the antibody binds. Thus if the antibody is monoclonal, the polypeptide will successfully compete with INGAP by virtue of it containing the epitope of the antibody. It may also be desirable that the antibodies be bound to a solid phase or support, such as polymeric beads, sticks, plates, etc.

Pharmaceutical compositions containing a mammalian INGAP protein may be used for treatment of pancreatic insufficiency. The composition may alternatively contain a polypeptide which contains a sequence of at least 15 consecutive amino acids of a mammalian INGAP protein. The polypeptide will contain a portion of INGAP which is biologically active in the absence of the other portions of the protein. The polypeptide may be part of a larger protein, such as a genetic fusion with a second protein or polypeptide. Alternatively, the polypeptide may be conjugated to a second protein, for example, by means of a cross-linking agent. Suitable portions of INGAP proteins may be determined by homology with amino acids #103 to #122 of SEQ ID NO:2, or by the ability of test polypeptides to stimulate pancreatic duct cells to grow and proliferate. As is known in the art, it is often the case that a relatively small number of amino acids can be removed from either end of a protein without destroying activity. Thus it is contemplated within

the scope of the invention that up to about 10% of the protein can be deleted, and still provide essentially all functions of INGAP. Such proteins have at least about 130 amino acids, in the case of hamster INGAP.

The pharmaceutical composition will contain a pharmaceutically acceptable diluent or carrier. A liquid formulation is generally preferred. INGAP may be formulated at different concentrations or using different formulants. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, dextrose, lactose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, alpha and beta cyclodextrin, soluble starch, hydroxyethyl starch and carboxymethylcellulose, or mixtures thereof. Sucrose is most preferred. Sugar alcohol is defined as a C_4 to C_8 hydrocarbon having an $-OH$ group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. Mannitol is most preferred. These sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to amount used as long as the sugar or sugar alcohol is soluble in the aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v % and 7.0 w/v %, more preferable between 2.0 and 6.0 w/v %. Preferably amino acids include levorotatory (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution, if these are used. Most any physiological buffer may be used, but citrate, phosphate, succinate, and glutamate buffers or mixtures thereof are preferred. Preferably, the concentration is from 0.01 to 0.3 molar. Surfactants can also be added to the formulation.

Additionally, INGAP or polypeptide portions thereof can be chemically modified by covalent conjugation to a polymer to increase its circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Pat. Nos. 4,766,106, 4,179,337, 4,495,285, and 4,609,546. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1000 and 40,000, more preferably between 2000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor.

After the liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients.

Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

The following examples are not intended to limit the scope of the invention, but merely to exemplify that which is taught above.

EXAMPLES

Example 1

This example describes the cloning and isolation of a cDNA encoding a novel, developmentally regulated, pancreatic protein.

We hypothesized that a unique locally produced factor(s) is responsible for islet cell regeneration. Using the recently developed mRNA differential display technique (5,6) to compare genes differentially expressed in cellophane wrapped (CW) versus control pancreata (CP) allowed us to identify a cDNA clone (RD19-2) which was uniquely expressed in cellophane wrapped pancreas.

A cDNA library was constructed from mRNA isolated from cellophane wrapped hamster pancreas using oligo d(T) primed synthesis, and ligation into pcDNA3 vector (Invitrogen). The number of primary recombinants in the library was 1.2×10^6 with an average size of 1.1 kb. The cDNA library was screened for clones of interest using high density colony plating techniques. Colonies were lifted onto nylon membranes (Schleicher & Schuell) and further digested with proteinase K (50(g/ml). Treated membranes were baked at 80° C. for 1 hour and hybridized at 50° C. for 16-18 hours with $1-5 \times 10^5$ cpm/ml of [32 P]-dCTP (Dupont-New England Nuclear) radiolabeled RD 19-2 probe. Colonies with a positive hybridization signal were isolated, compared for size with Northern mRNA transcript, and sequenced to confirm identity with the RD 19-2 sequence.

Example 2

This example compares the sequence of INGAP to other proteins with which it shares homology.

The nucleotide sequence of the hamster INGAP clone with the longest cDNA insert was determined. As shown in FIG. 1 the hamster cDNA comprises 747 nucleotides (nt), exclusive of the poly(A) tail and contains a major open reading frame encoding a 175 amino acid protein. The open reading frame is followed by a 3'-untranslated region of 206nt. A typical polyadenylation signal is present 11nt upstream of the poly(A) tail. The predicted INGAP protein shows structural homology to both the PAP/HIP family of genes which is associated with pancreatitis or liver adenocarcinoma (7-11) and the Reg/PSP/lithostatine family of genes (13,15) which has been shown to stimulate pancreatic beta-cell growth (14) and might play a role in pancreatic islet regeneration. Comparison of the nucleotide sequence and their deduced amino acids between hamster INGAP and rat PAP-I shows a high degree of homology in the coding region (60 and 58% in nucleotide and amino acid sequences, respectively). The predicted amino acid sequence of the hamster INGAP reveals 45% identity to PAP II and 50% to PAP III both of which have been associated with acute pancreatitis, and 54% to HIP which was found in a hepatocellular carcinoma. INGAP also shows 40% identity to the rat Reg/PSP/lithostatine protein (FIG. 2). Reg is thought to be identical to the pancreatic stone protein (PSP) (15,16) or pancreatic thread protein (PTP) (17). The N-terminus of the predicted sequence of INGAP protein is highly hydrophobic which makes it a good candidate for being the signal peptide

which would allow the protein to be secreted. Similar to PAP/HIP but different from the Reg/PSP/lithostatine proteins a potential N-glycosylation site is situated at position 135 of the INGAP sequence. Unique to INGAP is another potential N-glycosylation site situated at position 115. INGAP also shows a high degree of homology (12/18) (FIG. 2) with a consensus motif in members of the calcium-dependent (C-type) animal lectin as determined by Drickamer including four perfectly conserved cysteines which form two disulfide bonds (12). Two extra cysteines found at the amino-terminus of INGAP (FIG. 2) are also present in Reg/PSP and PAP/HIP. However, it is not clear what the biological significance might be.

Example 3

This example demonstrates the temporal expression pattern of INGAP upon cellophane-wrapping.

In order to determine the temporal expression of the INGAP gene, total RNA extracted from CP and CW pancreas was probed with the hamster INGAP cDNA clone in Northern blot analysis. A strong single transcript of 900 bp was detected (FIGS. 3A, 3B and 3C) 1 and 2 days after cellophane wrapping which disappeared by 6 through 42 days and was absent from CP. INGAP mRNA is associated with CW induced pancreatic islet neogenesis, since it is present only after CW. It is not likely that the increased expression of INGAP is associated with acute pancreatitis as is the case with the PAP family of genes. During the acute phase of pancreatitis the concentrations of most mRNAs encoding pancreatic enzymes including amylase are decreased significantly (16,18). In contrast, in the CW model of islet neogenesis in which high expression of INGAP has been detected, amylase gene expression was simultaneously increased above normal (FIGS. 3A, 3B and 3C) rather than decreased, suggesting that INGAP expression is not associated with pancreatitis but rather with islet neogenesis. The cause of increased amylase gene expression 1 and 2 days after CW is as yet unclear, and more studies need to be done to elucidate this issue. It is unlikely though, that the increase is associated with exocrine cell regeneration which occurs at a later time after CW (19). Thus, INGAP protein plays a role in stimulation of islet neogenesis, in particular, in beta cell regeneration from ductal cells.

Example 4

This example describes the cloning and partial sequence of a human cDNA encoding INGAP protein.

Human polyA⁺ RNA was isolated from a normal human pancreas using a commercially available polyA⁺ extraction kit from Qiagen. Subsequently, 500 ng polyA⁺ RNA was used as a template for reverse transcription and polymerase chain reaction (RT-PCR). The experimental conditions were set according to the instructions in the RT-PCR kit from Perkin Elmer. Oligo d(T) was used as the primer in reverse transcription. Primers corresponding to nucleotides 4 to 23 and 610 to 629 in SEQ ID NO:1 were used as the specific primers in the polymerase chain reaction. A 626 bp PCR fragment was cloned using a TA cloning kit from Invitrogen. The human INGAP cDNA is 100% identical to the hamster INGAP cDNA sequence in SEQ ID NO:1.

Example 5

This example demonstrates that synthetic peptides from INGAP play a role in stimulation of islet neogenesis, and that at least one epitope coded by the as yet unsequenced 120 bp segment of human INGAP is shared with hamster INGAP.

A synthetic peptide corresponding to amino acids 104-118 in SEQ ID NO:2 of the deduced hamster INGAP protein was used as an immunogen to raise polyclonal antibodies in a rabbit. The antiserum was subsequently used in immunohistochemistry assays using the avidin-biotin complex (ABC) method. Cells in the peri-islet region in humans with neo-islet formation stained positively for INGAP demonstrating that human and hamster INGAP share a common epitope between amino acids 104 to 118 in SEQ ID NO:2.

The same synthetic peptide was tested for its ability to stimulate ³H-thymidine incorporation into rat pancreatic tumor duct cells (ARIP) and hamster insulinoma tumor cells (HIT). 10 μ Ci of ³H-thymidine at 80.4 Ci/mole concentration was added to approximate 10⁶ cells cultured in Ham's F-12K media. After 24 hrs, the cells were harvested and solubilized. Differential precipitation of the nucleic acids with trichloroacetic acid (TCA) was performed according to the procedure modified by Rosenberg et al. and the ³H-thymidine proportion incorporated was calculated. Addition of the synthetic peptide to ARIP in culture resulted in a 2.4-fold increase in ³H-thymidine incorporation comparing to the absence of the synthetic peptide in the culture. The synthetic peptide had no effect on the control cell line HIT. This result strongly suggests that INGAP plays a role in stimulating islet neogenesis.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 7

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 747 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:

(A) ORGANISM: Cricetus

(i x) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 20..541

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

CTG CAAGACA GGTACCATG  ATG  CTT  CCC  ATG  ACC  CTC  TGT  AGG  ATG  TCT  TGG      52
      Met  Leu  Pro  Met  Thr  Leu  Cys  Arg  Met  Ser  Trp
      1                      5                      10
ATG  CTG  CTT  TCC  TGC  CTG  ATG  TTC  CTT  TCT  TGG  GTG  GAA  GGT  GAA  GAA      100

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Met	Leu	Leu	Ser	Cys	Leu	Met	Phe	Leu	Ser	Trp	Val	Glu	Gly	Glu	Glu	
			15					20					25			
TCT	CAA	AAG	AAA	CTG	CCT	TCT	TCA	CGT	ATA	ACC	TGT	CCT	CAA	GGC	TCT	148
Ser	Gln	Lys	Lys	Leu	Pro	Ser	Ser	Arg	Ile	Thr	Cys	Pro	Gln	Gly	Ser	
		30					35					40				
GTA	GCC	TAT	GGG	TCC	TAT	TGC	TAT	TCA	CTG	ATT	TTG	ATA	CCA	CAG	ACC	196
Val	Ala	Tyr	Gly	Ser	Tyr	Cys	Tyr	Ser	Leu	Ile	Leu	Ile	Pro	Gln	Thr	
	45					50					55					
TGG	TCT	AAT	GCA	GAA	CTA	TCC	TGC	CAG	ATG	CAT	TTC	TCA	GGA	CAC	CTG	244
Trp	Ser	Asn	Ala	Glu	Leu	Ser	Cys	Gln	Met	His	Phe	Ser	Gly	His	Leu	
	60				65				70						75	
GCA	TTT	CTT	CTC	AGT	ACT	GGT	GAA	ATT	ACC	TTC	GTG	TCC	TCC	CTT	GTG	292
Ala	Phe	Leu	Leu	Ser	Thr	Gly	Glu	Ile	Thr	Phe	Val	Ser	Ser	Leu	Val	
				80					85					90		
AAG	AAC	AGT	TTG	ACG	GCC	TAC	CAG	TAC	ATC	TGG	ATT	GGA	CTC	CAT	GAT	340
Lys	Asn	Ser	Leu	Thr	Ala	Tyr	Gln	Tyr	Ile	Trp	Ile	Gly	Leu	His	Asp	
		95						100					105			
CCC	TCA	CAT	GGT	ACA	CTA	CCC	AAC	GGA	AGT	GGA	TGG	AAG	TGG	AGC	AGT	388
Pro	Ser	His	Gly	Thr	Leu	Pro	Asn	Gly	Ser	Gly	Trp	Lys	Trp	Ser	Ser	
		110					115					120				
TCC	AAT	GTG	CTG	ACC	TTC	TAT	AAC	TGG	GAG	AGG	AAC	CCC	TCT	ATT	GCT	436
Ser	Asn	Val	Leu	Thr	Phe	Tyr	Asn	Trp	Glu	Arg	Asn	Pro	Ser	Ile	Ala	
	125					130					135					
GCT	GAC	CGT	GGT	TAT	TGT	GCA	GTT	TTG	TCT	CAG	AAA	TCA	GGT	TTT	CAG	484
Ala	Asp	Arg	Gly	Tyr	Cys	Ala	Val	Leu	Ser	Gln	Lys	Ser	Gly	Phe	Gln	
	140				145					150					155	
AAG	TGG	AGA	GAT	TTT	AAT	TGT	GAA	AAT	GAG	CTT	CCC	TAT	ATC	TGC	AAA	532
Lys	Trp	Arg	Asp	Phe	Asn	Cys	Glu	Asn	Glu	Leu	Pro	Tyr	Ile	Cys	Lys	
				160				165						170		
TTC	AAG	GTC	TAGGGCAGTT	CTAATTTCAA	CAGCTTGAAA	ATATTATGAA										581
Phe	Lys	Val														
GCTC	ACATGG	ACAAGGAAGC	AAGTATGAGG	ATTC	ACTCAG	GAAGAGCAAG	CTCTGCCTAC									641
ACACCCACAC	CAATTCCCTT	ATATCATCTC	TGCTGTTTTT	CTATCAGTAT	ATTCTGTGGT											701
GGCTGTAACC	TAAAGGCTCA	GAGAACAAAA	ATAAAATGTC	ATCAAC												747

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 174 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Leu	Pro	Met	Thr	Leu	Cys	Arg	Met	Ser	Trp	Met	Leu	Leu	Ser	Cys	
1				5					10					15		
Leu	Met	Phe	Leu	Ser	Trp	Val	Glu	Gly	Glu	Glu	Ser	Gln	Lys	Lys	Leu	
		20						25					30			
Pro	Ser	Ser	Arg	Ile	Thr	Cys	Pro	Gln	Gly	Ser	Val	Ala	Tyr	Gly	Ser	
		35					40					45				
Tyr	Cys	Tyr	Ser	Leu	Ile	Leu	Ile	Pro	Gln	Thr	Trp	Ser	Asn	Ala	Glu	
	50				55						60					
Leu	Ser	Cys	Gln	Met	His	Phe	Ser	Gly	His	Leu	Ala	Phe	Leu	Leu	Ser	
	65				70				75						80	
Thr	Gly	Glu	Ile	Thr	Phe	Val	Ser	Ser	Leu	Val	Lys	Asn	Ser	Leu	Thr	
			85					90					95			
Ala	Tyr	Gln	Tyr	Ile	Trp	Ile	Gly	Leu	His	Asp	Pro	Ser	His	Gly	Thr	
		100						105					110			

Leu	Pro	Asn 115	Gly	Ser	Gly	Trp	Lys 120	Trp	Ser	Ser	Ser	Asn 125	Val	Leu	Thr
Phe	Tyr 130	Asn	Trp	Glu	Arg	Asn 135	Pro	Ser	Ile	Ala	Ala 140	Asp	Arg	Gly	Tyr
Cys 145	Ala	Val	Leu	Ser	Gln 150	Lys	Ser	Gly	Phe	Gln 155	Lys	Trp	Arg	Asp	Phe 160
Asn	Cys	Glu	Asn	Glu 165	Leu	Pro	Tyr	Ile	Cys 170	Lys	Phe	Lys	Val		

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 175 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(i v) ANTI-SENSE: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: *Rattus rattus*

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met 1	Leu	His	Arg	Leu 5	Ala	Phe	Pro	Val	Met 10	Ser	Trp	Met	Leu	Leu 15	Ser
Cys	Leu	Met	Leu 20	Leu	Ser	Gln	Val	Gln 25	Gly	Glu	Asp	Ser	Pro 30	Lys	Lys
Ile	Pro	Ser 35	Ala	Arg	Ile	Ser	Cys 40	Pro	Lys	Gly	Ser	Gln 45	Ala	Tyr	Gly
Ser	Tyr 50	Cys	Tyr	Ala	Leu	Phe 55	Gln	Ile	Pro	Gln	Thr 60	Trp	Phe	Asp	Ala
Glu 65	Leu	Ala	Cys	Gln	Lys 70	Arg	Pro	Glu	Gly	His 75	Leu	Val	Ser	Val	Leu 80
Asn	Val	Ala	Glu	Ala 85	Ser	Phe	Leu	Ala	Ser 90	Met	Val	Lys	Asn	Thr 95	Gly
Asn	Ser	Tyr	Gln 100	Tyr	Ile	Trp	Ile	Gly 105	Leu	His	Asp	Pro	Thr 110	Leu	Gly
Gly	Glu	Pro 115	Asn	Gly	Gly	Gly	Trp 120	Glu	Trp	Ser	Asn	Asn 125	Asp	Ile	Met
Asn	Tyr 130	Val	Asn	Trp	Glu	Arg 135	Asn	Pro	Ser	Thr	Ala 140	Leu	Asp	Arg	Gly
Phe 145	Cys	Gly	Ser	Leu	Ser 150	Arg	Ser	Ser	Gly	Phe 155	Leu	Arg	Trp	Arg	Asp 160
Thr	Thr	Cys	Glu 165	Val	Lys	Leu	Pro	Tyr	Val 170	Cys	Lys	Phe	Thr	Gly 175	

(1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 175 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(v 1) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Leu Pro Pro Met Ala Leu Pro Ser Val Ser Trp Met Leu Leu Ser
1 5 10 15

-continued

Cys	Leu	Met	Leu	Leu	Ser	Gln	Val	Gln	Gly	Glu	Glu	Pro	Gln	Arg	Glu		
			20					25					30				
Leu	Pro	Ser	Ala	Arg	Ile	Arg	Cys	Pro	Lys	Gly	Ser	Lys	Ala	Tyr	Gly		
		35					40					45					
Ser	His	Cys	Tyr	Ala	Leu	Phe	Leu	Ser	Pro	Lys	Ser	Trp	Thr	Asp	Ala		
	50					55					60						
Asp	Leu	Ala	Cys	Gln	Lys	Arg	Pro	Ser	Gly	Asn	Leu	Val	Ser	Val	Leu		
65					70					75					80		
Ser	Gly	Ala	Glu	Gly	Ser	Phe	Val	Ser	Ser	Leu	Val	Lys	Ser	Ile	Gly		
			85						90					95			
Asn	Ser	Tyr	Ser	Tyr	Val	Trp	Ile	Gly	Leu	His	Asp	Pro	Thr	Gln	Gly		
		100						105						110			
Thr	Glu	Pro	Asn	Gly	Glu	Gly	Trp	Glu	Trp	Ser	Ser	Ser	Asp	Val	Met		
		115					120						125				
Asn	Tyr	Phe	Ala	Trp	Glu	Arg	Asn	Pro	Ser	Thr	Ile	Ser	Ser	Pro	Gly		
	130					135					140						
His	Cys	Ala	Ser	Leu	Ser	Arg	Ser	Thr	Ala	Phe	Leu	Arg	Trp	Lys	Asp		
145					150					155					160		
Tyr	Asn	Cys	Asn	Val	Arg	Leu	Pro	Tyr	Val	Cys	Lys	Phe	Thr	Asp			
				165					170					175			

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 174 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(v i) ORIGINAL SOURCE:

- (A) ORGANISM: Rattus rattus

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Leu	Pro	Arg	Val	Ala	Leu	Thr	Thr	Met	Ser	Trp	Met	Leu	Leu	Ser		
1				5					10					15			
Ser	Leu	Met	Leu	Leu	Ser	Gln	Val	Gln	Gly	Glu	Asp	Ala	Lys	Glu	Asp		
		20						25					30				
Val	Pro	Thr	Ser	Arg	Ile	Ser	Cys	Pro	Lys	Gly	Ser	Arg	Ala	Tyr	Gly		
		35					40					45					
Ser	Tyr	Cys	Tyr	Ala	Leu	Phe	Ser	Val	Ser	Lys	Ser	Trp	Phe	Asp	Ala		
	50					55					60						
Asp	Leu	Ala	Cys	Gln	Lys	Arg	Pro	Ser	Gly	His	Leu	Val	Ser	Val	Leu		
65					70					75					80		
Ser	Gly	Ser	Glu	Ala	Ser	Phe	Val	Ser	Ser	Leu	Ile	Lys	Ser	Ser	Gly		
			85						90					95			
Asn	Ser	Gly	Gln	Asn	Val	Trp	Ile	Gly	Leu	His	Asp	Pro	Thr	Leu	Gly		
		100						105						110			
Gln	Glu	Pro	Asn	Arg	Gly	Gly	Trp	Glu	Trp	Ser	Asn	Ala	Asp	Val	Met		
		115					120					125					
Asn	Tyr	Phe	Asn	Trp	Glu	Thr	Asn	Pro	Ser	Ser	Val	Ser	Gly	Ser	His		
	130					135					140						
Cys	Gly	Thr	Leu	Thr	Arg	Ala	Ser	Gly	Phe	Leu	Arg	Trp	Arg	Glu	Asn		
145					150					155					160		
Asn	Cys	Ile	Ser	Glu	Leu	Pro	Tyr	Val	Cys	Lys	Phe	Lys	Ala				
				165					170								

-continued

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 174 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Rattus rattus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met  Leu  Pro  Arg  Leu  Ser  Phe  Asn  Asn  Val  Ser  Trp  Thr  Leu  Leu  Tyr
 1      5      10
Tyr  Leu  Phe  Ile  Phe  Gln  Val  Arg  Gly  Glu  Asp  Ser  Gln  Lys  Ala  Val
 20     25     30
Pro  Ser  Thr  Arg  Thr  Ser  Cys  Pro  Met  Gly  Ser  Lys  Ala  Tyr  Arg  Ser
 35     40     45
Tyr  Cys  Tyr  Thr  Leu  Val  Thr  Thr  Leu  Lys  Ser  Trp  Phe  Gln  Ala  Asp
 50     55     60
Leu  Ala  Cys  Gln  Lys  Arg  Pro  Ser  Gly  His  Leu  Val  Ser  Ile  Leu  Ser
 65     70     75     80
Gly  Gly  Glu  Ala  Ser  Phe  Val  Ser  Ser  Leu  Val  Thr  Gly  Arg  Val  Asn
 85     90     95
Asn  Asn  Gln  Asp  Ile  Trp  Ile  Trp  Leu  His  Asp  Pro  Thr  Met  Gly  Gln
100    105    110
Gln  Pro  Asn  Gly  Gly  Gly  Trp  Glu  Trp  Ser  Asn  Ser  Asp  Val  Leu  Asn
115    120    125
Tyr  Leu  Asn  Trp  Asp  Gly  Asp  Pro  Ser  Ser  Thr  Val  Asn  Arg  Gly  Asn
130    135    140
Cys  Gly  Ser  Leu  Thr  Ala  Thr  Ser  Glu  Phe  Leu  Lys  Trp  Gly  Asp  His
145    150    155    160
His  Cys  Asp  Val  Glu  Leu  Pro  Phe  Val  Cys  Lys  Phe  Lys  Gln
165    170

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 165 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Rattus rattus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Met  Thr  Arg  Asn  Lys  Tyr  Phe  Ile  Leu  Leu  Ser  Cys  Leu  Met  Val  Leu
 1      5      10
Ser  Pro  Ser  Gln  Gly  Gln  Glu  Ala  Glu  Glu  Asp  Leu  Pro  Ser  Ala  Arg
 20     25     30
Ile  Thr  Cys  Pro  Glu  Gly  Ser  Asn  Ala  Tyr  Ser  Ser  Tyr  Cys  Tyr  Tyr
 35     40     45
Phe  Met  Glu  Asp  His  Leu  Ser  Trp  Ala  Glu  Ala  Asp  Leu  Phe  Cys  Gln
 50     55     60
Asn  Met  Asn  Ser  Gly  Tyr  Leu  Val  Ser  Val  Leu  Ser  Gln  Ala  Glu  Gly
 65     70     75     80
Asn  Phe  Leu  Ala  Ser  Leu  Ile  Lys  Glu  Ser  Gly  Thr  Thr  Ala  Ala  Asn
 85     90     95

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